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Sirna mediated gene silencing in transgenic animals

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SiRNA Mediated Gene Silencing in Transgenic Animals

The present invention relates to a process that enables constitutive and inducible gene knock down in living organisms using a short hairpin RNA expression vector integrated into the genome of the organism.

Background of the Invention

RNA interference (RNAi) has been discovered some years ago as a tool for inhibition of gene expression (Fire, A. et al., Nature 391, 806-811 (1998)). It based on the introduction of double stranded RNA (dsRNA) molecules into cells, whereby one strand is complementary to the coding region of a target gene. Through pairing of the specific mRNA with the introduced RNA molecule, the mRNA is degraded by a cellular mechanism. Since long dsRNA provokes an interferon response in mammalian cells, the technology was initially restricted to organisms or cells showing no interferon response (Bass, B.L., Nature 411, 428-429 (2001)). The finding that *short* (<30 bp) *interfering RNAs* (siRNA) circumvent the interferon response extended the application to mammalian cells (Elbashir, S.M. et al., Nature 411, 494-498 (2001)).

Although RNAi in mice has been in principle demonstrated, the current technology does not allow performing systematic gene function analysis *in vivo*. So far the inhibition of gene expression has been achieved by injection of *purified* siRNA into the tail vain of mice (McCaffrey, A.P. et al., Nature 418, 38-39 (2002); Lewis, D.H. et al., Nature Genet. 32, 107-108 (2002)). Using this approach, gene inhibition is restricted to specific organs and persists only a few days. A further improvement of the siRNA technology is based on the intracellular transcription of short hairpin RNA (shRNA) molecules using gene expression vectors (see Fig. 1; Brummelkamp, T.R. et al., Science 296, 550-553 (2002); Paddison, P.J. et al, Genes Dev. 16, 948-958 (2002); Yu, J.Y. et al., Proc. Natl. Acad. Sci. USA 99, 6047-6052 (2002); Sui, G. et al., Proc. Natl. Acad. Sci. USA 99, 5515-5520 (2002); Paul, C.P. et al., Nature Biotechnol. 20,

505-508 (2002); Xia, H. et al., Nat. Biotechnol. 10, 1006-10 (2002)). The activity of shRNA in mice has been demonstrated by McCaffrey et al., 2002 through injection of shRNA expression vectors into the tail vain. Again, gene inhibition was temporally and spatially restricted. Although these results demonstrate that the mechanism of shRNA mediated gene silencing is functional in mice, they do not clarify whether constitutive RNAi can be achieved in transgenic animals. Finally, Brummelkamp, T.R. et al., Science 296, 550-553 (2002) and Paddison, P.J. et al., Genes Dev. 16, 948-958 (2002), have shown the long-term inhibition of gene expression through stable integration of shRNA vectors in cultivated cell lines. These experiments included random integration of shRNA transgenes and screening for clones with appropriate siRNA expression, which is not applicable for testing of a large number of different shRNA transgenes in mice.

The *in vivo* validation of genes by RNAi mediated gene repression in a large scale setting requires the expression of siRNA at sufficiently high levels and with a predictable pattern in multiple organs. Targeted transgenesis provides the only approach to achieve reproducible expression of transgenes in the living organism (e.g. mammalians such as mice). It has been, however, questionable whether a single copy of a siRNA expression vector integrated into the genome would result in sufficiently high levels of siRNA required for RNAi-mediated gene inhibition in multiple organs of the living organism.

Most siRNA expression vectors are based on polymerase III dependent (Pol III) promoters (U6 or H1) that allow the production of transcripts carrying only a few non-homologous bases at their 3' ends. It has been shown that the presence of non-homologous RNA at the ends of the shRNA stretches lower the efficiency of RNAi mediated gene silencing (Xia et al., Nat. Biotechnol. 10, 1006-10 (2002)). However, it has been unpredictable for a person skilled in the art which genomic region within the living organism promotes an appropriate expression pattern of the Pol III promoter driven shRNA constructs required for RNAi-mediated gene inhibition in multiple organs of the living organism.

For the temporal control of RNAi mediated gene silencing in transgenic cells lines and living organism, a tight system for inducible siRNA expression is needed. Inducible gene expression systems based on the tetracycline dependent repressor are known. It is, however, not obvious whether a stably integrated, single copy configuration of these systems can be created that allows inducible RNAi in multiple organs without background activity.

Summary of the Invention

It has now been found that the modification of murine embryonic stem (ES) cells by the introduction of shRNA expression vectors through targeted transgenesis allows the inhibition of gene expression in mice generated from these modified ES cells. The invention thus provides

(1) the use of an expression vector comprising a short hairpin RNA (shRNA) construct under control of a ubiquitous promoter for preparing an agent for constitutive and/or inducible gene knock down (hereinafter also referred to as "RNAi mediated inhibition of gene expression") in a living organism or in cells of a cell culture;

(2) a method for constitutive and/or inducible gene knock down in a living organism or in cells of a cell culture which comprises stably integrating an expression vector as defined in (1) above into the genome of the living organism or into the cells of the cell culture;

(3) a living organism or cell culture having stably integrated, preferably at a polymerase II dependent locus of the living organism or cells of the cell culture, an expression vector as defined in (1) above; and

(4) an expression vector as defined in (1) above.

Short Description of the Figures

Fig. 1: Mechanism of RNAi through shRNA expression. A: expression of shRNA. B: shRNA processing. C: Pairing of siRNA with complementary mRNA strands. D: Cleavage of mRNA.

Fig 2: Pol III dependent shRNA expression vector (constitutive). Insertion of an shRNA expression vector into a ubiquitously expressed genomic locus.

Transcription through the upstream Pol II dependent promoter will be stopped by a synthetic polyadenylation signal (pA) and the hGH pA. The Pol III dependent promoter controls the expression of shRNA. The transcript is stopped by five thymidine bases.

Fig. 3: Tet repression system. tTS is a doxycycline controlled transcriptional silencer consisting of the tet repressor and the KRAB-AB domain of the Kid-1 protein. This fusion protein binds to the pol III dependent promoter via the tet operator sequences only in the absence of doxycycline and represses transcription.

Fig. 4: Vectors for Pol III dependent promoter based tet-repression system (inducible). Insertion of a shRNA expression vector into a ubiquitous expressed genomic locus. The transcription of the Pol II dependent promoter will be stopped by the synthetic polyadenylation signal (pA) and a hGH pA. An inducible Pol III dependent promoter controls the expression of shRNA. The transcript is stopped by five thymidine bases.

Fig. 5: Principle of the Doxycycline inducible gene expression system. rtTA2M2 is a doxycycline-controlled transcriptional activator consisting of the reverse tet repressor and the VP16 activation domain of the Herpes simplex virus VP16 protein. This fusion protein binds to a minimal CMV-promoter via the tet operator sequences in the presence of doxycycline and activates transcription. tTS functions in a similar fashion as explained in Fig. 3.

Fig. 6: Vectors for Pol II dependent promoter based tet-system (inducible). Insertion of a shRNA expression vector into a ubiquitous expressed genomic locus. The rtTA2M2 is expressed under the control of the ubiquitous Pol II dependent promoter, stopped by a synthetic polyadenylation signal (pA) and a hGH pA. An inducible Pol II dependent promoter controls the expression of shRNA. The transcript is stopped by a short polyadenylation signal.

Detailed Description of the Invention

The term "living organisms" according to the present invention relates to multi-cell organisms which can be vertebrates such as mammals (e.g. non-human animals such as rodents including mice and rats; and humans) or non-mammals (e.g. fish) or can be invertebrates such as insects or worms, or can be plants (higher plants, algi or fungi). Most preferred living organisms are mice and fish.

"Cell culture" according to the present invention includes cells isolated from the above defined living organisms and cultured *in vitro*. These cells can be transformed (immortalized) or untransformed (directly derived from living organisms; primary cell culture).

The expression vector according to the invention of the present application is suitable for stable integration into the living organism or into cells of the cell culture. It is moreover preferred that it contains homologous sequences suitable for targeted integration at a defined locus, preferably at a polymerase II dependent locus of the living organisms or cells of the cell culture.

The expression vector may further contain functional sequences selected from splice acceptor sequences (such as a splice acceptor of adenovirus, etc.), polyadenylation sites (such as synthetic polyadenylation sites, or the polyadenylation site of human growth hromones, etc.), selectable marker sequences (such as the neomycin phosphotransferase gene of *E. coli* transposon, etc.), etc.

The expression vector of the invention is suitable for the following particularly preferred approaches (for constitutive and inducible expression):

A. a Pol III dependent promoter (constitutive H1, U6) driven shRNA construct (to be integrated into a ubiquitously active Pol II dependent locus (see Fig. 2);

B. a Pol III dependent promoter (inducible U6) driven shRNA construct (to be integrated into a ubiquitously active Pol II dependent locus (Fig 3 and 4)); or

C. a polymerase II (Pol II) dependent promoter (inducible CMV) driven shRNA construct (to be integrated into a ubiquitously active Pol II dependent locus (Fig. 5 and 6)).

The ShRNA within the vector of the invention preferably comprises
(I) at least one DNA segment A-B-C wherein

A is a 15 to 35, preferably 19 to 29 bp DNA sequence being at least 95%, preferably 100% complementary to the gene to be knocked down (e.g. firefly luciferase, p53, etc.);

B is a spacer DNA sequence having 5 to 9 bp forming the loop of the expressed RNA hair pin molecule, and

C is a 15 to 35, preferably 19 to 29 bp DNA sequence being at least 85% complementary to the sequence A; and
(II) a stop and or polyadenylation sequence.

A preferred embodiment of the method (2) of the invention concerns the following steps:

1. Generation of the short hairpin DNA containing the antisense- and sense-strand of the coding region of a gene (e.g. firefly luciferase; p53). Antisense and sense-strand are separated by a spacer of 5 to 9 bp.
2. Generation of constructs for the expression of the above mentioned shRNA under the control of a constitutive or inducible promoter (Pol II or Pol III dependent).
3. Insertion of the mentioned expression constructs into a ubiquitous expressed Pol II locus by homologous recombination in ES cells.
4. Analysis of the constitutive and inducible inhibition of gene expression (e.g. firefly luciferase; p53) in ES cells (e.g. through Western blot analysis).

5. Generation of mice using the mentioned ES cells and analysis of the inhibition of gene expression in several tissues (e.g. firefly luciferase; p53; e.g. through Western blot analysis).

The procedure's advantages over current technology are as follows:

(i) The stable and body wide inhibition of gene expression by generating transgenic animals (such as mice).

(ii) The reversible inhibition of gene expression using the inducible constructs.

CLAIMS:

1. Use of an expression vector comprising a short hairpin RNA construct under control of a ubiquitous promoter for preparing an agent (shRNA) for constitutive and/or inducible gene knock down in a living organism or in cells of a cell culture.

2. The use of claim 1 wherein the expression vector
(i) is suitable for stabile integration into the genome of a living organism or into the genome of cells of the cell culture; and/or
(ii) contains homologous sequences suitable for integration at a defined genomic locus through homologous recombination, preferably at a polymerase II dependent locus in the genome of the living organism or in the genome of the cells of the cell culture including embryonic stem cells; and/or
(iii) further contains functional sequences selected from splice acceptor sequences, polyadenylation sites, selectable marker sequences, etc.

3. The use of claim 1 or 2 wherein
(i) the ubiquitous promoter is selected from polymerase I, II and III dependent promoters and preferably is a polymerase II or III dependent promoter; and/or
(ii) the ubiquitous promoter is constitutive or inducible; and/or
(iii) the living organism is a multi-cell organism including vertebrates, invertebrates and plants and the cell culture is derived from said living organism.

4. The use according to any one of claims 1 to 3 wherein the expression vector is

(a) a Pol III dependent promoter (constitutive H1, U6) driven shRNA construct suitable to be integrated into a ubiquitously active Pol II dependent locus;

(b) a Pol III dependent promoter (inducible U6) driven shRNA construct suitable to be integrated into a ubiquitously active Pol II dependent locus; or

(c) a Pol II dependent promoter (inducible CMV) driven shRNA construct suitable to be integrated into a ubiquitously active Pol II dependent locus.

5. The use according to any one of claims 1 to 4 wherein the shRNA comprises (I) at least one DNA segment A-B-C wherein

A is a 15 to 35, preferably 19 to 29 bp DNA sequence with at least 95%, preferably 100% complementarily to the gene to be knocked down;

B is a spacer DNA sequence having 5 to 9 bp forming the loop of the expressed RNA hair pin molecule, and

C is a 15 to 35, preferably 19 to 29 bp DNA sequence with at least 85% complementarily to the sequence A; and

(II) a stop and or polyadenylation sequence.

6. A method for constitutive and/or inducible gene knock down in a living organism or in cells of a cell culture which comprises stably integrating an expression vector as defined in any one of claims 1 to 5 into the genome of the living organism or into the cells of the cell culture.

7. The method of claim 6 wherein the expression vector is integrated at a polymerase locus of the living organism or cell culture.

8. The method of claim 6 or 7, wherein the method for constitutive and/or inducible gene knock down in a living organism comprises integrating the expression vector into ES cells of the living organism.

9. A living organism or cell culture having stably integrated, preferably at a polymerase II dependent locus of the living organism or cells of the cell culture, an expression vector as defined in any one of claims 1 to 5.

10. An expression vector as defined in any one of claims 1 to 5.

Abstract

The present invention relates to a process that enables constitutive and inducible gene knock down in living organisms using a short hairpin RNA expression vector integrated into the genome of the organism.

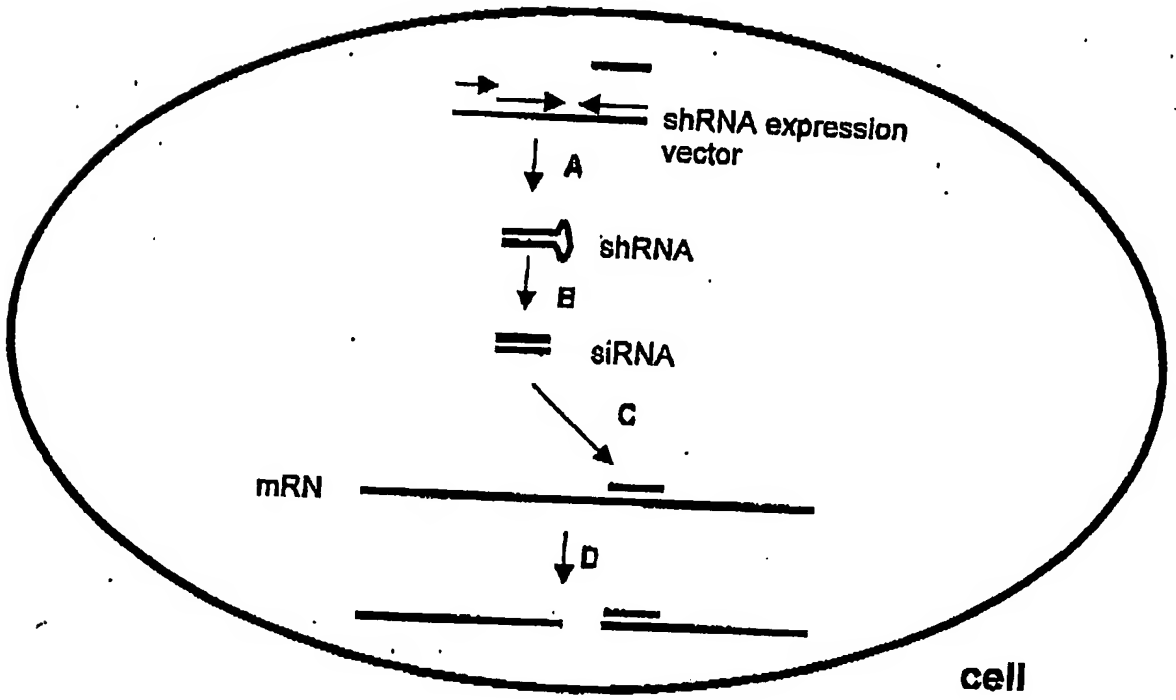
shRNA expression vector
A
shRNA
B
siRNA
C
mRN
D
cell

Fig.1

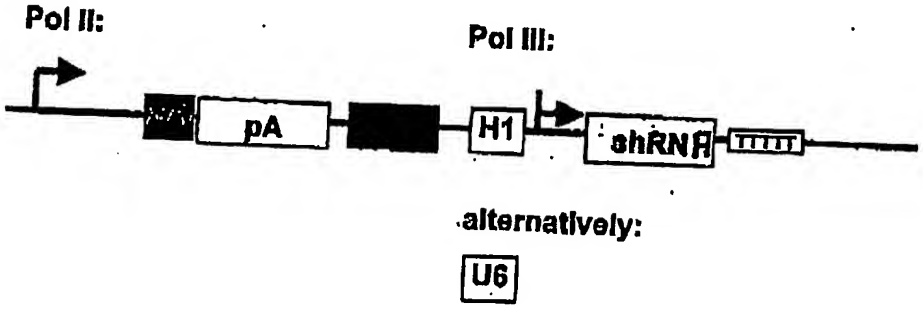
Pol II:
Pol III:
pA
H1
shRNA
alternatively:
U6

Fig.2

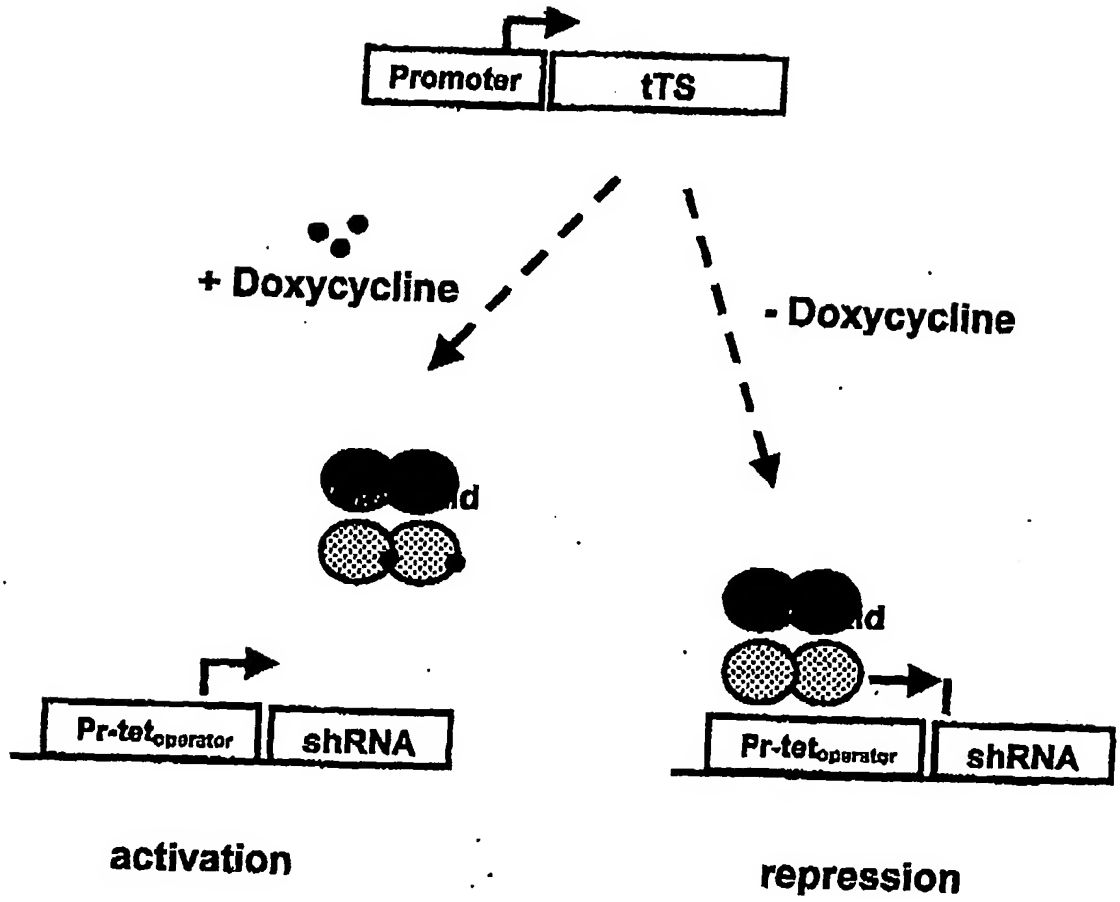
Promoter
tTS
+ Doxycycline
- Doxycycline
Pr-tet operator
shRNA
Pr-tet operator
shRNA
activation
repression

Fig.3

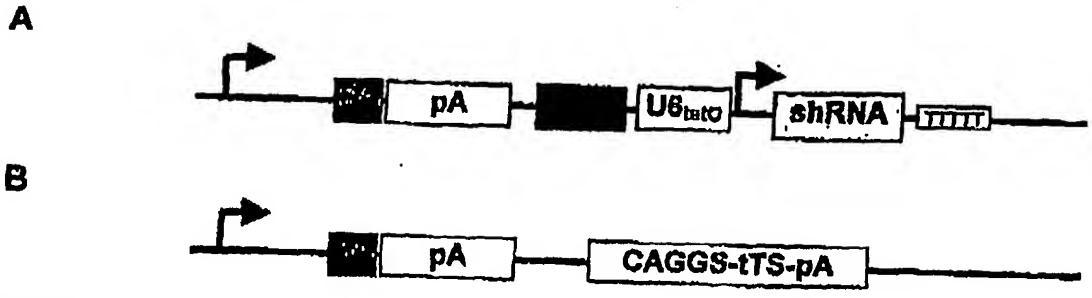
A
pA
U6 tetO
shRNA
B
pA
CAGGS-tTS-pA

Fig.4

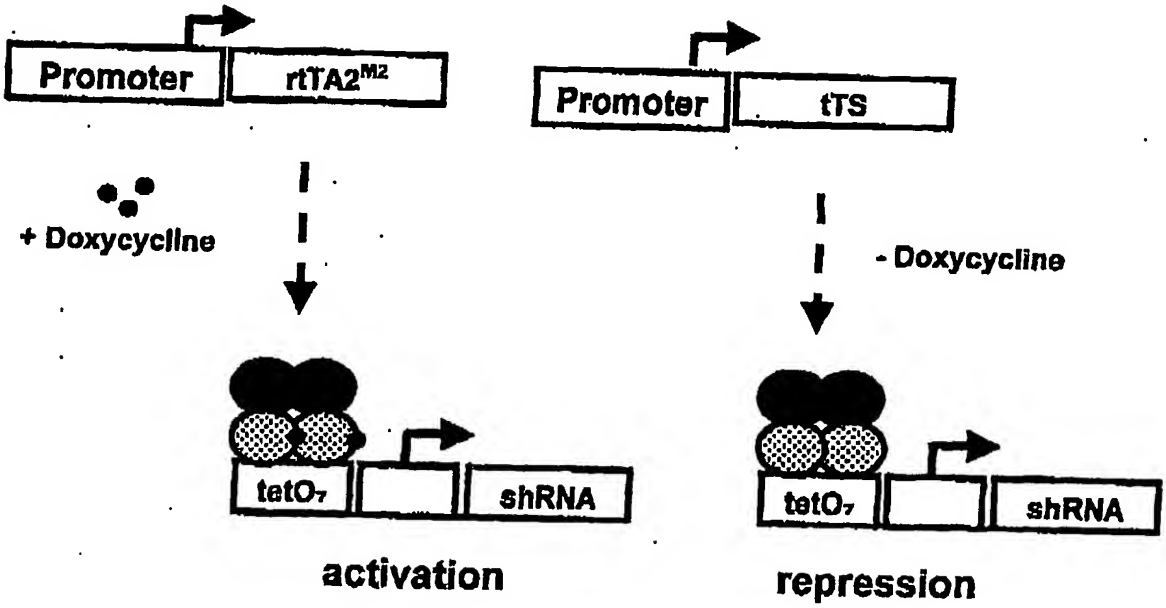
Promoter
rtTA2M2
+ Doxycycline
tetO7
shRNA
activation
Promoter
tTS
- Doxycycline
tetO7
shRNA
repression

Fig.5

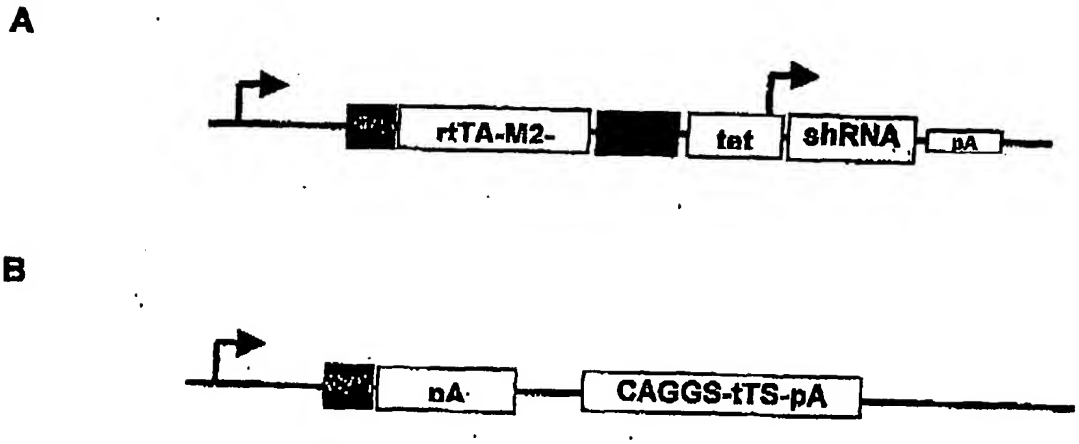
A
rtTA-M2-
tet
shRNA
pA
B
pA
CAGGS-tTS-pA

Fig.6